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AN 1987:134093 CAPLUS
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TI Detection of GLO-I isozymes in human blood by a rapid method
AU Sehajpal, Prabodh K.; Sandhu, Harpreet S.; Tewari, Kusum; Sharma, Vijay K.
CS Dep. Hum. Biol., Punjabi Univ., Patiala, 147 002, India
SO Ann. Biol. (Ludhiana, India) (1986), 2(1), 90-2
CODEN: ANBIEO
DT Journal
LA English
CC 7-1 (Enzymes)
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COMPLETED

SHORT COMMUNICATIONS

Annals of Biology, Vol. 2, No. 1, pp. 90—92, 1986

Detection of GLO-I Isozymes in Human Blood by a Rapid Method

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Kompf *et al.* (1975a) reported a genetic marker, red cell glyoxalase-I (GLO-I) which catalyzes the conversion of reduced glutathione and methyl glyoxal to S-lactoyl glutathione (Racker, 1951; Crook and Law, 1952; Rose, 1957). GLO-I polymorphism is under genetic control having two alleles GLO^1 and GLO^2 set at an autosomal locus (Kompf *et al.*, 1975b). Separation of GLO-I types has been achieved using starch gel (Kompf *et al.*, 1975a; Parr *et al.*, 1977; Emes and Parkin, 1980), agarose gel (Kuhnl *et al.*, 1977) and mixed starch-cum-agarose gel electrophoresis (Scott and Fowler, 1982). However, these methods are expensive and time consuming. In this study we report a rapid method for the detection of red cell GLO-I isozymes from human blood.

A random blood sample of 100 Jat Sikhs born and residing in the Patiala city was collected. Fresh haemolysates were prepared from washed (×3) red blood cells by freezing and thawing. Haemolysates were stored at −20°C until analysed. Before electrophoresis the haemolysates were treated with 0.2M β-mercaptoethanol for 20 min. GLO-I typing was done by modifying the earlier method of Hashimoto *et al.* (1978). Tank buffer (pH 7.4) had 0.1 M Tris, 0.034 M citric acid, 0.039 M boric acid and 0.25 M lithium hydroxide. Gel buffer was prepared by 1 : 10 dilution of the tank buffer with 0.02 M magnesium chloride. A 1.2 mm thick gel was prepared with starch (0.5% w/v) and agarose 1% (w/v) in the gel buffer. Slits were made in the gel with the help of 3 mm Whatman paper. β-Mercaptoethanol treated haemolysates were loaded in the slits and electrophoresis was carried out at a constant voltage of 10 V/cm at 4°C for 2 h.

For staining, the reaction mixture containing 0.25 ml of 25% methyl glyoxal and 20 mg reduced glutathione in 5 ml 0.2 M phosphate buffer (pH 6.8) was applied on the gel surface as filter paper overlay. The gel and the paper were incubated for

30 min at 37°C. After incubation, excess of the reaction mixture was removed with dry filter paper and the gel was immersed in a developing mixture containing 100 mg iodine and 200 mg potassium iodide in 100 ml distilled water and kept at room temperature for 10 min until blue bands appeared against colourless background. In addition to this modified method, haemolysates were also typed by the methods of Kompf *et al.* (1975a) and Scott and Fowler (1982).

Table 1 presents the distribution of the GLO-I phenotypes in one hundred blood samples. The allele frequencies estimated for GLO^1 and GLO^2 were 0.220 and 0.780 respectively. The frequency here is in conformity with the reported data on north Indian populations (Ghosh, 1977). No difficulty was encountered for typing haemolysates with all the three mentioned techniques and the results obtained were unequivocal.

Table 1. Phenotype and gene frequency of GLO-I isozymes among Jat Sikhs

Sample size		Phenotypes 1-1	2-1	2-2	Gene frequency
100	Observed	6	32	62	GLO^1=0.220
	Expected	4.84	34.32	60.84	GLO^2=0.780

It was observed that the method of Kompf *et al.* (1975a) requiring 14 h for a single run, is good enough for GLO-I typing, but has the disadvantage of time-consuming electrophoretic run and also requires ample amount of haemolysates for correct typing. Scott and Fowler (1982) described a new media, starch-cum-agarose gel for GLO-I typing, making use of L-histidine monochloride in the gel buffer. The resolution of the bands was achieved in 2.5 h.

In the method described here, the main emphasis was to avoid the use of L-histidine monochloride, either in the gel buffer or in the tank buffer, as the compound is known to degrade rapidly in high voltage electrophoresis (Scott and Fowler, 1982). Keeping this and the advantage of mixed starch/agarose media in view Mg^{++} ions were added to the gel buffer of Hashimoto *et al.* (1978) for the detection of red cell glyoxalase-I types. Parr *et al.* (1977) and Stohlmacher and Haferland (1980) had observed that the inclusion of Mg^{++} ions gives a greater rate of reaction and more linear response in production of S-lactoyl glutathione in spectrophotometric assay of GLO-I. It is already known that such a modification helps especially in typing haemolysates stored for long periods.

Our method requires only about 2h for the resolution of GLO-I bands and a small amount of haemolysates for analysis. In addition, the chemicals consumed are much cheaper compared with methods of Kompf *et al* (1975a) and Scott and Fowler (1982). The method will be very useful in the mass screening programmes and for the laboratories having small budgets.

ACKNOWLEDGEMENT

KT is thankful to the Bureau of Police Research and Development for financial assistance.

REFERENCES

Crook, E. M. and Law, K. (1952) Glyoxalase : the role of the components. *Biochemistry* **52** : 492.

Emes, E. G. and Parking, B. H. (1980) The determination of glyoxalase I phenotypes in human blood stains. *Forens. Sci. Int.* **15** : 265-271.

Ghosh, A. K. (1977) Polymorphism of red cell glyoxalase I. *Hum. Genet.* **39** : 91-95.

Hashimoto, M.; Harada, S. and Omoto, K. (1978) Red cell glyoxalase I polymorphism in Japanese : Confirmation of a low GLO^{-1}I frequency. *Jap. J. Hum. Genet.* **23** : 139-143.

Kompf, J.; Bissbort, S.; Gussmann, S. and Ritter, H. (1975a) Polymorphism of red cell glyoxalase I (EC : 4.4.1 5). A New genetic marker in man. *Humangenetik* **27** : 141-143.

Kompf, J.; Bissbort, S. and Ritter, H. (1975b) Red cell glyoxalase I (EC : 4.4.1.5): formal genetics and linkage relations. *Hum. Genet.* **28** : 249-251.

Kuhnl, P.; Schwabenl, R. and Spielman, W. (1977) Investigation on the polymorphism of the glyoxalase I in the population of Hassen, Germany. *Hum. Genet.* **38** : 99.

Parr, C. W.; Bageter, I. A. and Welsch, S. G. (1977) Human red cell glyoxalase I polymorphism. *Biochem. Genet.* **15** : 109-113.

Racker, E. (1951) The mechanism of action of glyoxalse. *J. Biol. Chem.* **190** : 685.

Rose, I. A. (1957) Mechanism of the action of glyoxalase I. *Acta Biochem. Biophys.* **25** : 214.

Scott, A. G. and Fowler, J. C. S. (1982) Electrophoretic typing of glyoxalase I (GLO-I) isozymes using a mixed starch/agrose gel. *Forens. Sci. Int.* **20** : 287-294.

Stohlmacher, P. and Haferland, W. (1980) Glyoxalase I in human tissues. *Zeit. Rechtsmed.* **25** : 165.

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09/728,378 Biotech (2)1771.445

L8 ANSWER 10 OF 92 CAPLUS COPYRIGHT 2002 ACS
AN 1993:434404 CAPLUS
DN 119:34404
TI Agarose gel electrophoresis system for the separation of antibiotics used in animal agriculture
AU Salvatore, Michael J.; Feygin, Ilya; Katz, Stanley E.
CS Merck and Co., Inc., Rahway, NJ, 07065, USA
SO Analyst (Cambridge, United Kingdom) (1993), 118(3), 281-7
CODEN: ANALAO; ISSN: 0003-2654
DT Journal
LA English

Agarose Gel Electrophoresis System for the Separation of Antibiotics used in Animal Agriculture

Michael J. Salvatore and Ilya Feygin
Merck & Co., Inc. P.O. Box 2000, Rahway, NJ 07065, USA
Stanley E. Katz
Department of Biochemistry and Microbiology, Cook College/NJAES, Rutgers—the State University of New Jersey, New Brunswick, NJ 08903-0231, USA

A novel electrophoresis system using agarose gel has been developed for the separation and as an aid in the classification of antibiotics. This system utilizes Nunc cell factory disposable tissue culture dishes, which serve as bioassay dish and cooling chamber for agarose gel, in a custom designed electrophoresis unit. Tris(hydroxymethyl) methylamine–succinate buffer at pH 6.0 and 8.0 are employed as the electrolyte for electrophoresis. Bioautography was used as the indicator of mobility. Any agar diffusion assay can be modified to use this system. A suggested name for this system is Nunc cell factory agarose gel electrophoresis (NUAGE). Selected antibiotics, representative of the aminoglycoside, β-lactam, macrolide, moenocinol, peptide, polyene, polyether, quinone and tetracycline classes, were separated with this system.

Keywords: *Agarose gel electrophoresis; animal agriculture; bioautography; separation of antibiotics; classification of antibiotics*

The current official, Association of Official Analytical Chemists (AOAC), methods of analysis rely primarily upon agar diffusion assays for the quantification of antibiotic levels in feeds.[1] These methods assume that the declared antibiotic is solely responsible for the biological response. These assays also assume a prior knowledge of the constituents of the feed. For example, an assay for erythromycin would be used to analyse feeds assumed to contain erythromycin. The key to error in these assays is the 'assumption' of the identity of the antibiotic prior to its analysis. There is no provision to identify the antibiotic in this type of assay because the results are demonstrated only by zones of inhibition.

The AOAC methods do not address the analysis of antibiotics occurring in mixtures. They cannot, *per se*, detect, separate or differentiate between individual, classes or mixtures of antibiotics. The design of these assays can completely mask the presence of other antibiotics. This is also true for combinations of antibiotics that act in synergy or as antagonists with each other; all of which can lead to erroneous and misleading results.

It is, therefore, important to develop an antibiotic identification and classification system. Such a system should provide rapid detection with the simplicity of an agar diffusion assay and a means of detecting separating and identifying individual classes and mixtures of antibiotics. A separation system designed to differentiate between antibiotics can be achieved by various means, some of which include: (*i*) exploitation of some chemical or physical property of the antibiotic molecule (*i.e.*, charge, size); (*ii*) selective destruction or inhibition of the activity of the antibiotic (*i.e.*, pH change, chemical inactivation); (*iii*) selective indication of individual antibiotics (*i.e.*, colour reaction, spectra); and (*iv*) selectivity by indirect means (*i.e.*, resistant microorganisms). To date, only separation by chemical or physical property has shown any merit.

There have been many classification systems based on chemical and/or physical properties described for the detection, separation and identification of antibiotics. These systems have been described for paper chromatography,[2–10] thin-layer chromatography,[11–18] paper electrophoresis[19–25] and gel electrophoresis.[26–35] All of these systems utilize differential solubility and/or mobility to detect, separate and identify antibiotics.

Paper and thin-layer chromatography systems take advantage of the antibiotic solubility and differential mobility in a solvent for classification. These systems use multiple transfer steps, solvents and support matrices to separate and characterize antibiotics into discernible groups. Low levels of antibiotics have to be concentrated prior to testing. If bioautography is used to indicate mobility and biological activity there is also a drying and antibiotic diffusion step.

In addition, complex mixtures can contain constituents (salts, fatty acids, *etc.*) that interefere usually by causing streaking or yield R_F (retardation factor) values that are not reproducible.[2–18] These types of systems are tedious and time consuming, ineffective for all antibiotics, insensitive and can expose the antibiotic to harsh conditions.

The aforementioned chromatography systems are very useful for organic-soluble antibiotics. Aqueous-soluble antibiotics, such as tetracyclines, aminoglycosides, *etc.*, are very difficult to characterize due to their lack of mobility.

Paper and gel electrophoresis utilize charge, shape and size to separate and characterize antibiotic molecules. Differential mobilities of antibiotic molecules in an electric field can be used for characterization. Systems have been described that utilize organic solvents, such as chloroform,[36] with paper electrophoresis and aqueous solvents, such as phosphate buffer, with agarose gel electrophoresis.[6,16–20,22,24–30]

Paper electrophoresis is more suitable for antibiotic residue analysis than it is for chromatography. It is a simpler and more direct method of analysis. It can handle organic- as well as aqueous-soluble antibiotics for characterization. However, this type of system contains the same insensitivity, overloading, streaking, drying and antibiotic diffusion problems as chromatography. In addition, electroendoosmosis can further affect the accuracy of the characterization.

Gel electrophoresis is, by far, the best of the separation systems. Overloading, and thus sensitivity can be minimized by using thicker support layers without increasing electroendoosmosis. Streaking can be minimized by decreasing the amount of gel comprising the matrix. Finally, drying and antibiotic diffusion problems are eliminated by overlaying the gel with a biological indicator contained in a similar type of matrix.

The gel electrophoresis system presented herein detects,

separates, classifies and identifies 17 antibiotics currently used in animal agriculture when used in conjunction with the solvent separation system of Salvatore and Katz.[23]

Experimental

Reagents

The reagent ingredients were dissolved in de-ioinized water, the pH was adjusted and the total volume made up 1 l.

Tris–succinate buffer, pH 6.0. Tris(hydroxymethyl)methylamine (Tris; 1.82 g) and 0.95 g succinic acid.

Tris–succinate buffer, pH 8.0. Tris(hydroxymethyl)methylamine (3.03 g) and 0.85 g succinic acid.

Sterile isotonic saline solution. Sodium chloride (9.0 g). Sterilize for 20 min at 121 °C.

1% *Agarose–Tris–succinate, pH* 6.0. Agarose (10.0 g) and 1.0 l of Tris–succinate buffer pH 6.0. Sterilize for 20 min at 121 °C. After sterilization agarose–Tris–succinate was kept at 48 °C until use.

1% *Agarose–Tris–succinate, pH* 8.0. Agarose (10.0 g) and 1.0 l of Tris–succinate buffer pH 8.0. Sterilize for 20 min at 121 °C. After sterilization agarose–Tris–succinate was kept at 48 °C until use.

Culture Media

The media described below were re-hydrated in 1 l of distilled water, adjusted to the appropriate pH and autoclaved at 121 °C for 20 min. Media containing agar were kept in a water-bath at 48 °C until use.

Medium A. Antibiotic medium 3 (53.0 g) and 0.1 g manganese(ous) sulfate H_2O (Fisher Scientific, Springfield, NJ, USA), pH 6.95–7.05.

Medium B. Nutrient broth (8.0 g) and 2.0 g yeast extract, pH 6.95–7.05.

Medium C. Medium B and 15.0 g Bacto-Agar, pH 6.95–7.05.

Medium D. Yeast extract (10.0 g) and 10.0 g anhydrous dextrose (Fisher Scientific), pH 6.55–6.65.

Medium E. Medium D and 15.0 g Bacto-Agar, pH 6.55–6.65.

All biological media were purchased from Difco Laboratories, Detroit, MI, USA).

Antibiotics

Antibiotics were chosen because of their use in animal agriculture in the USA. Their preparation is outlined in the AOAC manual.[1] Fosfomycin and L-proline (no antiobiotic activity) were used as indicators of mobility. Fosfomycin was prepared by following the same procedure as that for streptomycin. The following antibiotics were used in these studies: bacitracin, zinc salt; bambermycin; chlortetracycline hydrochloride; erythromycin; fosfomycin, disodium; hygromycin; lincomycin hydrochloride; monensin, sodium salt; neomycin sulfate; novobiocin, sodium salt; nystatin; oleandomycin, phosphate salt; oxytetracycline dihydrate; penicillin G, sodium salt; spectinomycin dihydrochloride; streptomycin sulfate; tylosin tartrate; virginiamycin. All antibiotics except bambermycin, fosfomycin and virginiamycin were purchased from Sigma, St. Louis, MO, USA. Bambermycin was obtained from Hoechst Pharmaceuticals, Somerville, NJ, USA, virginiamycin was obtained from Smith Kline and Beckman, Paoli, PA, USA. Fosfomycin was obtained from Merck, P.O. Box 2000, Rahway, NJ, USA.

Culture Maintenance and Preparation

Bacillus subtilis

Bacillus subtilis [American Type Culture Collection (ATCC) 6633] was prepared according to the procedures outlined in the AOAC manual.[1] Spore suspensions were sub-divided into 2 ml aliquots (≈6 × 10^8 viable spores per ml) and stored at 4 °C. Overlays were prepared by dilution of 0.5 ml of spore suspension with 4.5 ml of medium B. Five ml of diluted spore suspension were added per 100 ml of molten medium C.

Saccharomyces cerevisiae

Saccharomyces cerevisiae (ATCC 9763) was prepared according to the procedures outlined in the AOAC manual.[1] A fresh culture was used each day to prepare overlays. For the overlays, a fresh broth culture was diluted with medium D to 70% transmission at 660 nm in a Spectronic 20 spectrophotometer (Fisher Scientific). Four millilitres of this culture was added per 100 ml of molten medium E.

Electrophoretic Gels

Two hundred ml of agarose–Tris–succinate buffer at either pH 6.0 or 8.0 (see Reagents section) was dispensed onto the top support shelf of a Nunc cell factory [Laboratory Disposable Products (Springfield, NJ, USA); Fig 1]. Air bubbles were removed by flaming with a bunsen burner and the agarose was allowed to solidify on a levelling table. After solidifying, 950 ml of a 30% solution of antifreeze, coolant and water was dispensed into the bottom chamber of the cell factory. The gel was cooled to 4 °C in a refrigerator and stored until use.

Electrophoresis

Equivalent volumes (about 750 ml) of a solution of Tris–succinate buffer at 4 °C was dispensed into each buffer trough

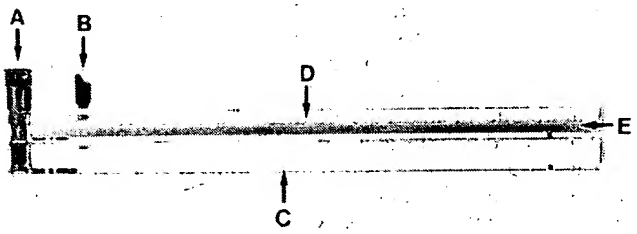

Fig. 1 Side view of a Nunc cell factory containing an agarose gel layer. A, Coolant outlet; B, coolant inlet; C, cooling chamber; D, agarose layer; and E, support shelf

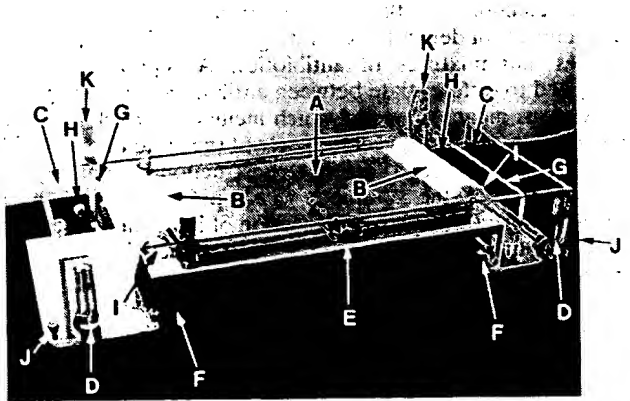

Fig. 2 Corner view of electrophoretic chamber (base) containing Nunc cell factory, paper wicks and wick supports. A, Nunc cell factory; B, paper wicks; C, buffer troughs; D, electrodes; E, cell factory support; F, trough connector; G, trough baffle; H, trough outlet (to pump); I, wick support; J, levelling screw; and K, cover support

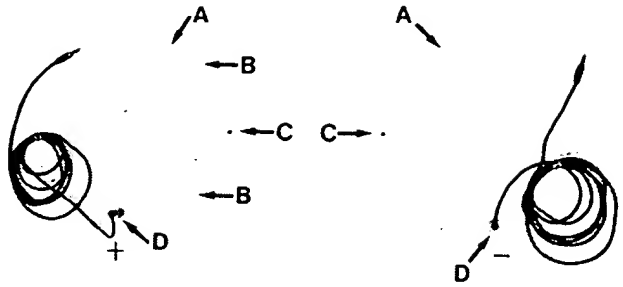

Fig. 3 Top view of cover for electrophoretic chamber with electrode wire leads. A, Vacuum ports; B, inlet and outlet ports; C, air vent controls; and D, electrode wire leads

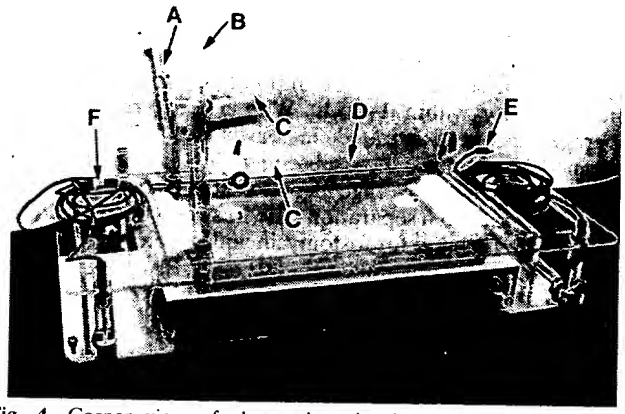

Fig. 4 Corner view of electrophoretic chamber with cover and cooling connector valves. A, Inlet connector valve; B, outlet connector valve; C, purge valve; D, chamber cover; E, cathode wire lead; and F, anode wire lead

in the electrophoresis chamber (Fig. 2). A circulating pump [Master Flex variable speed pump, Cole Parmer (Chicago, IL, USA)] connected to both buffer troughs was used to recirculate electrolyte (about 10 rev min^{-1}). A cell factory containing agarose–Tris–succinate gel at the appropriate pH was placed on the gel support between the two buffer troughs. Two glass wick supports, one at each end of the cell factory, were also placed on the gel support. Four mm wells were then punched into the centre of the gel about 2.5 cm apart with a gel punch [Bio-Rad Laboratories (Richmond, CA, USA)]. Twenty microlitres of sample to be assayed was pipetted into each well. To track the migration from the origin and current flow an indicator dye (Bromocresol Green, 10% solution) was pipetted into the centre well for each determination. Paper wicks [15.25 × 14 cm (6 × 5½ in) Whatman filter paper No. 3] were soaked in buffer and placed about 1 cm from each end of the gel, on top of the wick support and into the buffer trough.

The chamber cover (Fig. 3) was placed on top of the electrophoretic chamber that connected the buffer troughs to a computer controlled power supply (Bio-Rad Laboratories). To remove excess condensate, the cover was also connected to a vacuum line *via* rubber tubing and a liquid trap.

The cooling chamber of the cell factory was connected to a refrigerated water circulator (Bio-Rad Laboratories) *via* an inlet and outlet valve (Fig. 4). The circulator was brought to the appropriate temperature, the air purged from the coolant lines and the feed and return valves opened. The coolant was allowed to circulate until the appropriate gel temperature was achieved. The chamber was connected to the power supply and a constant voltage applied to the gel for the specified time.

Bioautography

After electrophoresis, the cell factories were removed from the electrophoresis chamber, the coolant was poured out of the bottom chamber and water at 50 °C was dispensed into the bottom chamber. The gel was allowed to reach 48 °C. The cell factory was then placed on a level table and 150 ml of the appropriate inoculated medium (see Culture Maintenance and Preparation Section) was poured over the top of the agarose layer and allowed to solidify. The water in the bottom layer was poured out of the bottom chamber, the plate was covered and incubated at the appropriate temperature for 18 h (or overnight).

Mobility was demonstrated by measuring the location of the zones of inhibition. The distance from the origin to the beginning of the zone, plus one half the diameter, along with the position of the zone was recorded.

Electroendoosmosis

Twenty microlitres of a 5 mg ml^{-1} aqueous solution of L-proline was pipetted into each well of a cell factory and assayed electrophoretically under the appropriate conditions. A methanol solution of 1% ninhydrin was then pipetted into each well and along each lane of the gel. These gels were warmed for 1 h by filling the bottom chamber of the cell factory with 48 °C water. A yellow colour appeared where there was a presence of L-proline. L-Proline was assayed in each position five times and the results were averaged.

Results and Discussion

Design of Electrophoretic Equipment

Gel matrix and support

Many types of gel matrices have been described for use in electrophoresis; some of which are better suited than others for specific applications. Polyacrylamide and starch gels are more suitable for protein separations whereas agar and agarose are typically used for the separation of smaller molecules. Therefore, it is imperative to use a gel that has a consistent composition (batch-to-batch) and lacks interfering components.

The amount of agarose typically used in these types of gels ranges from 1 to 2%.[6,16–20,24–30,32–35] However, a 1% concentration was chosen in this system to minimize any residual electroendoosmotic effects and allow easier spreading. Concentrations of greater than 1% led to stiffer gels that had a tendency to clump and solidify before a uniform layer could be spread. Concentrations of less than 1% led to softer gels that accumulated water during electrophoresis and the results were not reproducible. The amount of prepared agarose used per plate was 200 ml, which gave a gel 4 mm in thickness. Volumes less than this generally led to clumping and uneven gels.

Cooling is one of the major problems in electrophoresis. Uneven cooling can lead to poor separations. Therefore, a means to consistently cool gel layers during electrophoresis is of primary importance. Nunc cell factories, which are usually used for the proliferation of tissue/cell cultures, were selected as cooling chambers. A side view of a cell factory loaded with agarose gel prior to electrophoresis is shown in Fig. 1. It consists of a sealed chamber with inlet (A) and outlet (B) valves and a 1 cm lip on top of the chamber. The top part of the chamber and the top lip can be used as a dish to support the gel layer. It is also level from one end to the other to allow pouring of a gel layer that is flat and of uniform thickness. The chamber normally used for growing tissue culture can be filled with antifreeze and the inlet and outlet valves connected to a chiller/recirculator, which will circulate coolant, at constant temperature, to the chamber and ultimately cool the gel layer.

Electrophoretic chamber

It was necessary to design a chamber in which the electrophoresis could be performed. This chamber is shown in Fig. 2. It consists of: a shelf (E), which will support the cell factory/agarose gel; and two buffer troughs (C); which contain

baffles (G) and platinum wire electrodes (D). These troughs were situated at either end of the shelf with enough space to allow the placement of wick supports (I). To eliminate suction effects, a tube (F) was placed between the troughs connecting them and levelling screws (J) were placed on the base. Electrolyte gradients were also eliminated by connecting trough outlets (H) with tygon tubing to a pump circulating electrolyte between troughs. The height of the support shelf and the outside of the electrophoretic chamber were adjusted to accommodate the connection of the troughs and the agarose gel with paper wicks (B). Finally, supports (K) for the cover were placed opposite the electrodes preventing motion.

bThe cover for the chamber is seen in Fig. 3. It is designed to cover the distance between the buffer troughs completely. It has two ports (B) that fit over the inlet and outlet that connect the cell factory to the chiller/recirculator. It also has two vacuum ports (A) for the removal of moisture and any gas generated during electrophoresis. These vacuum ports are connected to a vacuum trap *via* rubber tubing. To control air flow into and ultimately out of the chamber, two air vent controls (C) are placed in the centre of the cover. Finally, two wire leads (D) with female to male connectors were attached to the lid, the female connectors mated exactly with the male connectors on the chamber. Connection to a power pack is made *via* these leads. Orientation of the poles can be in either direction.

It was also necessary to develop a valve system to connect the cell factory to the chiller/recirculator. This was accomplished by a loop of tygon tubing with branch points. Each branch point carried coolant to and from the electrophoretic chamber. This tubing was connected (Fig. 4) to an inlet connector valve (A), which had a bypass valve (C) to purge any air before it could enter the coolant chamber of the cell factory. The outlet connector valve (B) was connected to tubing that returned the coolant to the chiller/recirculator. It also has a purge valve (C) to remove unwanted air from the system.

Electrophoretic Conditions

In order to obtain consistent results from each electrophoretic separation, it was necessary to standardize the system. Each parameter had to be examined to maximize resolution and maintain consistency.

Before such parameters were examined, it was necessary to determine the type of electrolyte to be used. This electrolyte would be used in the aqueous as well as the solid phase of the system and would be selected such that any background interference would be minimized. For this reason citrate (divalent cation chelator) and phosphate (cation chelator and antibiotic uptake inhibitor) buffers were eliminated as electrolyes.[37,38] Smither and Vaughan[35] developed a system that utilized Tris–succinate at pH 6.0 and 8.0 to separate antibiotics in agar and agarose gel and which had no background interference. Therefore, Tris–succinate was the electrolyte of choice. It is also important to determine mobility as related to pH as Smither and Vaughan[35] have demonstrated. The pH values chosen, 6.0 and 8.0, were sufficiently spread to allow differentiation between the antibiotics and still lie in the range of biological activity.

The best means of applying the sample to the gel had to be determined. It was apparent that a well should be used as a reservoir for the sample. This would minimize spreading and the diffusion of the antibiotic into the gel. The well size should be sufficiently small not to affect migration but large enough to hold a concentration of antibiotic that could be detected. A well size of 4 mm was chosen. Sensitivity and reproducibility problems were seen with smaller well sizes. Well sizes of larger than 4 mm resulted in migration and reproducibility problems.

Finally, it was necessary to select a marker that could be used to determine maximum migration. The antibiotics fosfomycin and streptomycin were chosen as indicators of mobility. Fosfomycin is one of the fastest migrating anionic antibiotics whereas streptomycin is one of the fastest migrating cationic antibiotics. They were used to standardize electrophoretic conditions. It was also decided to use bioautography to track the mobility of these anitibiotics. *B. subtilis* was used as an indicator of the activity and thus mobility.

Volumes of agar of less than 150 ml, when used as an overlay on the agarose gel, solidified too quickly causing clumping and uneven thicknesses of the agar layer.

Time

Run time was studied under the premise that this system should be simple and rapid. Thus, the faster the run time the simpler and quicker the assay. The results of various run times at pH 6.0 and 8.0 for fosfomycin and streptomycin are summarized in Table 1. Run times of 1.0, 1.5 and 2.0 h were chosen. The results indicate that a 1 h run time was not sufficient for separation. The antibiotics tested attain maximum velocity within 1–1.5 h. With a 1.5 h run time, the average distance migrated increased to 127% over the distance migrated in 1 h. A subsequent increase in run time to 2 h led only to a 3% average increase in the distance migrated. Therefore, the 1.5 h separation time was chosen as the modest increases in mobility for fosfomycin and streptomycin did not warrant the extra time taken for 2 h.

Temperature

Temperature is another parameter that must be controlled to maintain consistency and accuracy. Other workers have indicated that a temperature range of 10 to 20 °C should be maintained during electrophoresis.[26–35] With modern temperature controllers, it is easier to regulate the system temperature. Therefore, a narrower range of temperature can be maintained, resulting in increased accuracy and reproducibility.

The results of electrophoresis performed on fosfomycin and streptomycin at 10, 15 and 20 °C are summarized in Table 2. Overall a 1 °C increase in temperature resulted in an increase

Table 1 Electrophoretic mobility (values given in cm) of streptomycin and fosfomycin at various times. (Conditions: 20 V cm^{-1}, 10 °C. *Bacillus subtilis* was used as the biological indicator of activity)

	Time/h		
Antibiotic	1.0	1.5	2.0
pH 6.0—			
Fosfomycin	a* 3.2	a 7.1	a 73
Streptomycin	c‡ 2.7	c 5.2	c 5.6
pH 8.0—			
Fosfomycin	a 4.5	a 10.4	a 10.6
Streptomycin	c 3.3	c 5.8	c 6.0

* a = Anion.
‡ c = Cation.

Table 2 Electrophoretic mobility (values given in cm) of streptomycin and fosfomycin at various temperatures. (Conditions: 20 V cm^{-1}, 1.5 h *Bacillus subtilis* was used as the biological indicator of activity)

	Temperature/°C		
Antibiotic	10	15	20
pH 6.0—			
Fosfomycin	a* 7.1	a 7.9	a 8.6
Streptomycin	c† 5.2	c 5.7	c 6.3
pH 8.0—			
Fosfomycin	a 10.4	a 11.1	a 12.3
Streptomycin	c 5.8	c 6.5	c 7.2

* a = Anion.
† c = Cation.

Table 3 Electrophoretic mobility (values given in cm) of streptomycin and fosfomycin at various voltages. (Conditions: 10 °C, 1.5 h. *Bacillus subtilis* was used as the biological indicator of activity. For voltages of 25 and 30 V cm^{-1} paper wicks were attached to the agarose gel)

	Voltage/V cm^{-1}		
Antibiotic	20	25	30
pH 6.0—			
Fosfomycin	a* 7.1	a 10.2	a 13.6
Streptomycin	c† 5.2	c 9.6	c 13.9
pH 8.0—			
Fosfomycin	a 10.4	ND‡	ND
Streptomycin	c 5.8	c 11.0	ND

* a = Anion.
† c = Cation.
‡ ND = No activity detected.

Table 4 Electrophoretic mobility (values given in cm) of streptomycin and fosfomycin at 25 V cm^{-1} and various times. (Conditions: 10 °C, 25 V cm^{-1}. *Bacillus subtilis* was used as the biological indicator of activity. For all times paper wicks were attached to the agarose gel)

	Time/h		
Antibiotic	0.5.	1.0	1.5
pH 6.0—			
Fosfomycin	a* 5.0	a 7.5	a 10.2
Streptomycin	c† 4.8	c 7.2	c 9.6
pH 8.0—			
Forfomycin	a 7.5	a 11.1	ND‡
Streptomycin	c 5.0	c 7.6	c 11.0

* a = Anion.
† c = Cation.
‡ ND = No activity detected.

in mobility of 2.1% at pH 6.0 and 1.8% at pH 8.0 for fosfomycin. With streptomycin, a 2.1% increase was seen at pH 6.0. At pH 8.0 there was a 2.4% increase in mobility. However, for streptomycin at both pH 6.0 and 8.0, tailing was observed with increases in temperature. At 15 °C a 1.0 cm tail and at 20 °C a 2.5 cm tail was observed. The results indicate that although there is enhanced separation at increased temperatures tailing also increases. Therefore, it would be best to run separations at 10 °C.

Voltage

The voltage used per centimetre of gel (the driving force) is another parameter that will affect the mobility and accuracy of the system. It is very important to use a power source that can provide constant current. Lightbown and de Rossi[32] and Smither and Vaughan[35] suggested votlages of between 20 and 30 V cm^{-1}.

The results of electrophoresis performed on fosfomycin and streptomycin at 20, 25 and 30 V cm^{-1} are summarized in Table 3 and the results of electrophoresis of fosfomycin and streptomycin at 25 V cm^{-1} at 0.5, 1.0 and 1.5 h are shown in Table 4. The data indicated that 20 V cm^{-1} was the maximum voltage that should be used with this system. At voltages of 25 and 30 V cm^{-1} the paper wicks adhered to the agarose gel making them difficult to remove. It must also be kept in mind that any increase in voltage will result in a quadratic increase in the heat generated. Thus, the lower the voltage the easier it is to regulate cooling.

Electroendoosmosis

When designing any electrophoretic separation system, it is important to consider the effects that electroendoosmosis will have on the migration of an ion. Typically, electroendoosmosis will cause a shift in migration towards the cathode; the extent of which is a property of the support matrix. Electroendoosmosis was monitored at both pH 6.0 and 8.0 utilizing L-proline as the migrating ion and ninhydrin as the indicator of mobility. These results indicate slight migration (0.2 cm) toward the cathode at both pH 6.0 and 8.0. Electroendoosmotic effects were the same at both pHs. Although this migration is negligible, 0.2 cm should be used as a correction factor, when determining the exact mobilities of antibiotics. Correction factors should be determined using the individual apparatus.

Table 5 Electrophoretic mobility of selected antibiotics. (Conditions: 1.5 h, 20 V cm^{-1}, 10 °C. *Bacillus subtilis* was used as the biological indicator for all antibiotics except nystatin; *Saccharomyces cerevisiae* was the biological indicator for nystatin. Mobility of each antibiotic was adjusted for electroendoosmotic drift)

		Mobility/cm	
Antibiotic	Class	pH 6.0	pH 8.0
Erythromycin	Macrolide	c* 2.5	c 3.1
Oleandomycin	Macrolide	c 2.5	c 3.0
Tylosin	Macrolide	c 1.2	c.1.3
Hygromycin b	Aminoglycoside	c 5.2	c 5.0
Neomycin	Aminoglycoside	c 5.5	c 5.5
Streptomycin	Aminoglycoside	c 5.2	c 5.8
Lincomycin	Aminoglycoside-like	c 3.0	c 2.6
Spectinomycin	Aminoglycoside-like	c 6.0	c 5.0
Chlortetracycline	Tetracycline	c 1.6	a† 1.5
Oxytetracycline	Tetracycline	c 1.3	a 1.3
Bacitracin	Peptide	c 1.6	a 0.5
Virginiamycins	Peptide-like	c 1.3	a 2.3/a 0.8
Bambermycins	Moenocinol	a 3.8/a 2.4	a 2.3/a 1.6
Monensin	Polyether	a 1.4	a 1.3
Novobiocin	Quinone	a 1.4	a 2.4
Nystatin	Polyene	c 0.6	c 0.6
Penicillin G	β-Lactam	a 2.7	a 3.8

* c = Cation.
† a = Anion.

Electrophoretic Mobility of Antibiotics

The results of the electrophoretic mobilities of the 17 antibiotics tested are given in Table 5. The macrolide antibiotics (erythromycin, oleandomycin and tylosin) migrated as cations. Erythromycin and oleandomycin migrated the furthest and showed similar mobilities at both pH 6.0 and 8.0. These two antibiotics also demonstrated an increase in mobility with an increase in pH.

The aminoglycoside class of antibiotics (hygromycin B, neomycin and streptomycin) migrated as cations and have similar mobilities. At pH 6.0, neomycin migrated the furthest of these antibiotics, to 5.5 cm. At pH 8.0, however, streptomycin migrated the furthest with an increase in mobility to 5.8 cm, followed by neomycin, which had no enhancement of mobility, and hygromycin B, which showed a decrease of mobility to 5.0 cm.

The aminoglycoside-like class of antibiotics (lincomycin and spectrinomycin) also migrated as cations with lincomycin migrating 3.0 cm and spectrinomycin 6.0 cm, at pH 6.0. Both antibiotics demonstrated a decrease in mobility with increasing pH.

The tetracycline class of antibiotics (chlortetracycline and oxytetracycline) migrated as cations at pH 6.0, with chlortetracycline being the fastest of the two antibiotics. At pH 8.0, however, these two antibiotics reversed direction and migrated as anions with chlortetracycline being the faster of the two antibiotics.

The peptide and peptide-like antibiotics (bacitracin and virginiamycin) migrated toward the cathode at pH 6.0; bacitracin migrated the furthest. With a change in pH, both of

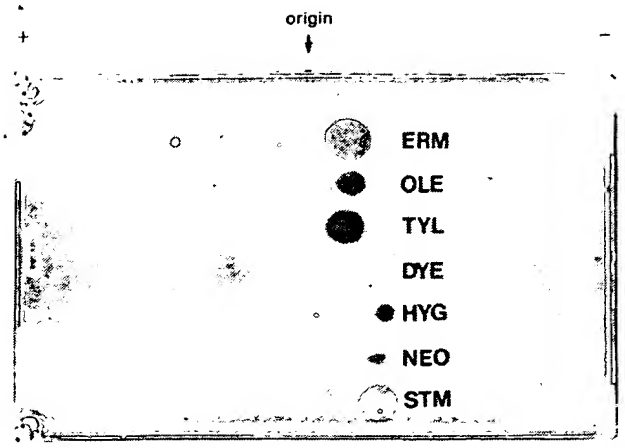

Fig. 5 Top view of a pH 6.0 agarose gel containing an indicator dye and various antibiotics after electrophoresis and bioautography. ERM, Erythromycin; OLE, oleandomycin; TYL, tylosin; HYG, hygromycin B; NEO, neomycin; and STM, streptomycin. *Bacillus subtilis* was used as the biological indicator

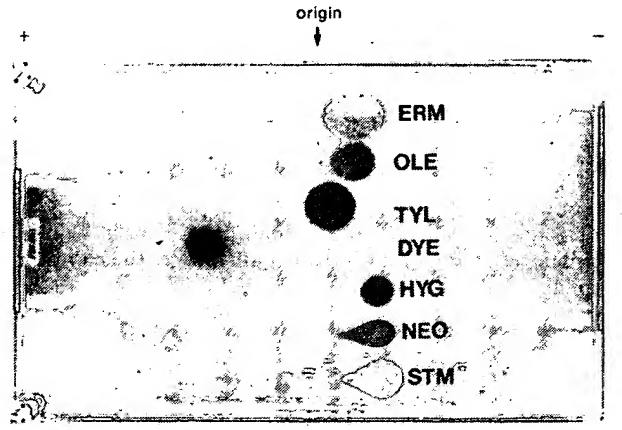

Fig. 6 Top view of a pH 8.0 agarose gel containing an indicator dye and various antibiotics after electrophoresis and bioautography. ERM, Erythromycin; OLE, oleandomycin; TYL, tylosin; HYG, hygromycin B; NEO, neomycin; and STM, streptomycin. *Bacillus subtilis* was used as the biological indicator

these antibiotics acted as anions with virginiamycins migrating the furthest. Virginiamycins at this pH also separates into two components, both of which migrated further than bacitracin.

The peptide-like antibiotics (bacitracin and virginiamycins) migrated toward the cathode at pH 6.0; bacitracin migrated the furthest. With a change in pH, both of these antibiotics acted as anions with virginiamycins migrating the furthest. Virginiamycins at this pH also separates into two components, both of which migrated further than bacitracin.

Also summarized in Table 5 are the electrophoretic mobilities of the miscellaneous class of antibiotics. Bambermycin demonstrated two components at both pH 6.0 and 8.0. Both components migrated toward the anode and demonstrated a decrease in mobility with an increase in pH. Monensin acted as an anion and migrated toward the anode. The distance migrated decreased slightly with an increase in pH. Novobiocin also acted as an anion by migrating to the anode. Unlike monensin, it demonstrated an increase in mobility when the pH was raised to 8.0. Nystatin exhibited some slight cationic activity and did not demonstrate any difference in migration from one pH to another. Penicillin G migrated towards the anode at both pH 6.0 and 8.0. It displayed an increase in mobility with an increase in pH.

Examples of the typical biological responses seen after electrophoresis and bioautography for the macrolide and aminoglycoside antibiotics at pH 6.0 and 8.0 are shown in Figs. 5 and 6, respectively. Also demonstrated are the enhancements in zone size with increasing pH.

Table 6 Selected electrophoretic conditions for the separation of antibiotics. (Conditions were selected that will maximize separation while maintaining efficiency)

Parameter	Condition
Time	1.5 h
Temperature	10 °C
Voltage	20 V cm^{-1}
Electrolyte	Tris–succinate; pH 6.0 and 8.0
Matrix	Agarose; 1.0%
Matrix thickness	4 mm; 200 ml per dish
Sample volume	40 µl
Sample receptable	Well; 4 mm
Overlay thickness	3 mm; 150 ml per dish

Conclusion

The conditions that maximize separation of antibiotics while maintaining efficiency are summarized in Table 6. This system represents an efficient and easy to perform method for the separation and identification of classes of antibiotics and, in some cases, individual antibiotics of the same class.

This system eliminates the insensitivity, overloading, streaking and antibiotic diffusion problems associated with chromatography and paper electrophoresis. It has the ability to separate and characterize antibiotics that are usually affected by harsh conditions (drying, solvent exposure, *etc.*). Agar diffusion assays currently in use for antibiotic testing can be adapted to this system. This system has been used for the quantitative and qualitative analysis of 17 antibiotics used in animal feeds.[39] Finally, elements of this system are disposable, Nunc cell factories make gel handling very easy and assay plates can be disposed of like any other disposable bioassay dish.

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References

1 *Official Methods of Analysis of the Association of Official Analytical Chemists*, ed. William, S., 15th edn., Association of Official Analytical Chemists, Arlington, VA, 1990, sect. 957.23-975.61.
2 Betina, V., *Nature (London)*, 1958, **182**, 796.
3 Betina, V., *J. Chromatogr.*, 1964, **15**, 379.
4 Betina, V., in *Methods in Enzymology*, ed. Hash, J. H., Academic, New York, 1975, vol. 43, pp. 101–172.
5 Betina, V., and Nemec, P., *Nature (London)*, 1960, **187**, 1111.
6 Goodall, R. R., and Levi, A. A., *Nature (London)*, 1946, **158**, 675.
7 Macek, K., in *Chromatography: A Laboratory Handbook of Chromatographic and Electrophoretic Methods*, ed. Heftmann, E., 3rd edn., Van Nostrand Reinhold, New York, 1975, pp. 139–163.
8 Wagman, G. H., and Bailey, J. V., *J. Chromatogr.*, 1969, **41**, 263.
9 Wagman, G. H., and Weinstein, M. J., *Chromatography of Antibiotics*, Elsevier, New York, 1973, pp. 23–405.
10 Wagman, G. H., and Weinstein, M. J., *Chromatography of Antibiotics*, 2nd edn., Elsevier, New York, 1984, pp. 23–504.
11 Aszalos, A., Davis, S., and Frost, D., *J. Chromatogr.*, 1968, **37**, 487.
12 Aszalos, A., and Frost, D., in *Methods in Enzymology*, ed. Hash, J. H., Academic, New York, 1968, vol. 18, 172–213.
13 Betina, V., *J. Antibiot. Ser. A*, 1964, **17**, 127.
14 Ikekawa, T., Iwami, F., Akita, E., and Umezawa, H., *J. Antibiot. Ser. A*, 1963, **16**, 56.
15 Ito, Y., Namba, M., Nagahama, N., Yamaguchi, T., and Okuda, T., *J. Antibiot., Ser. A*, 1964, **17**, 218.

16 Stahl, E., in *Chromatography: A Laboratory Handbook of Chromatographic and Electrophoretic Methods*, ed. Heftmann, E., 3rd edn., Van Nostrand Reinhold, New York, 1975, pp. 164–188.
17 Synder, L. R., in *Chromatography: A Laboratory Handbook of Chromatographic and Electrophoretic Methods*, ed. Heftmann, E., 3rd edn., Van Nostrand Reinhold, New York, 1975, pp. 46–76.
18 Salisbury, C. D. C., Rigby, C. E., and Chan, W., *J. Agric. Food Chem.*, 1989, **37**, 105.
19 Audubert, R., and de Mende, S., *The Principles of Electrophoresis*, Hutchinson, London, 1959, pp. 1–120.
20 Lederer, M., *An Introduction to Paper Electrophoresis and Related Methods*, Elsevier, New York, 1957, pp. 1–193.
21 Maede, K., Yagi, A., Naganawa, H., Kondo, S., and Umezawa, H., *J. Antibiot.*, 1969, **22**, 635.
22 Michl, H., in *Chromatography: A Laboratory Handbook of Chromatographic and Electrophoresis Methods*, ed. Heftmann, E., 3rd edn., Van Nostrand Reinhold, New York, 1975, pp. 282–311.
23 Salvatore, M. J., Jr., and Katz, S. E., *J. Assoc. Off. Anal. Chem.*, 1988, **71**, 1101.
24 Umezawa, H., and Kondo, S., in *Methods in Enzymology*, ed. Hash, J. H., Academic, New York, 1975, vol. 43, pp. 279–291.
25 Vondracek, M., in *Chromatography: A Laboratory Handbook of Chromatographic and Electrophoretic Methods*, ed. Heftmann, E., 3rd edn., Van Nostrand Reinhold, New York, 1975, pp. 815–840.
26 Freidlin, P. J., *Avian Dis.*, 1988, **32**, 370.
27 Giddings, J. C., in *Chromatography A Laboratory Handbook of Chromatographic and Electrophoretic Methods*, ed. Heftmann, E., 3rd edn., Van Nostrand Reinhold, New York, 1975, pp. 27–45.
28 Grynne, B., *Acta Pathol. Microbiol. Scand., Sect. B: Microbiol.*, 1973, **8**, 583.
29 Horng, H., and Ko, H., *Proc. Natl. Sci. Counc., Repub. China, Part 2*, 1977, **10**, 321.
30 Horng, C., Hsieh, J., Ko, H., Jan, R., and Li, J., *Proc. Natl. Sci. Counc., Repub. China*, 1979, **3**, 382.
31 Jorgenson, J. W., *Anal. Chem.*, 1986, **58**, 743A.
32 Lightbown, J. W., and de Rossi, P., *Analyst*, 1965, **90**, 89.
33 Lott, A. F., and Vaughan, D. R., *Soc. Appl. Bacteriol. Tech. Ser.*, 1983, **18**, 331.
34 Lott, A. F., Smither, R., and Vaughan, D. R., *J. Assoc. Off. Anal. Chem.*, 1985, **68**, 1018.
35 Smither, R., and Vaughan, D. R., *J. Appl. Bacteriol.*, 1978, **44**, 421.
36 Whitaker, J. R. in *Paper Chromatography and Electrophoresis*, ed. Zweig, G., and Whitaker, J. R., Academic, New York, 1967, vol. I, pp. 1–356.
37 Cassidy, P. J., 1990, personal communication.
38 Zimmerman, S. B., 1990, personal communication.
39 Salvatore, M. J., and Katz, S. E., *J. Assoc. Off. Anal. Chem.*, in the press.

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PROTEOGLYCAN ELECTROPHORESIS ON HORIZONTAL SUBMERGED POLYACRYLAMIDE-AGAROSE GELS

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A method of proteoglycan electrophoresis on submerged horizontal polyacrylamide-agarose gels is described. Several preparations of purified proteoglycans extracted from fetal and young baboon articular cartilage and from mandibular dog-fish cartilage were analyzed. Discrete bands corresponding to proteoglycan monomers of different size were obtained. The results were similar to those obtained using the more tedious electrophoretic separation on cylindrical gel rods.

INTRODUCTION

Incorporation of agarose for support gives mechanical stability to gels of low polyacrylamide content and large pore size allowing gel electrophoretic analysis of high molecular compounds. The composite agarose-acrylamide gels were first used for gel electrophoretic analysis of high molecular weight RNA (1)(2). A method using composite rod gels for glycosaminoglycan and proteoglycan analysis was subsequently described (3). Various proteoglycan preparations were analyzed using slightly modified variants of this method (4,5,6,7). The main result of these latter studies was the finding that cartilage proteoglycan monomers can be separated in several populations which yield discrete bands on electrophoresis. Previous attempts to prepare discrete fractions of proteoglycans by gel chromatography or equilibrium density centrifugation had been unsuccessful due probably to the polydispersity and overlaping of proteoglycan populations in these systems. The gel electrophoretic studies showed that the proteoglycan populations present age, species and anatomical site dependent variations (4) (7) (8) (9) and recently several cartilage proteoglycan populations which yield on gel electrophoresis single bands were isolated and characterized (10) (11) (12). The gel electrophoretic method was also used to analyze proteoglycans obtained from small biopsies of growth cartilage and several abnormalities were found in chondrodysplastic patients (13,14). Composite slab gel electrophoresis was more rarely used and no details of the methods were published (15) (16). The present communication describes a method for proteoglycan separation by electrophoresis on composite slab gels using an horizontal system for submerged gels.

MATERIALS AND METHODS

Materials

Guanidine hydrochloride was from Schwarz Mann, benzamidine HCl, 6-aminohexanoic acid, sodium EDTA and CsCl were from Merck; Sepharose CL-4B and CL-2B were from Pharmacia; DEAE cellulose (DE-52, microgranular form) was from Whatman; acrylamide and bisacrylamide were from Canalco; agarose (indubiose A 37) was from Industrie Biologique Francaise. Urea (for biochemistry grade) was from Merck. Film Gelbond was from FMC, Marine Colloids Division.

Proteoglycan Preparation

a) Tissues — The large joints of young baboons (Papio papio, Institut Pasteur, Garches, France) were removed immediately after death, frozen in solid CO_2 and stored at −40°C. After thawing to 4°C, the cartilage was separated from non-cartilaginous structures, diced finely with a scalpel and washed briefly in ice cold 0.9% NaCl. A baboon foetus (about 4.5 months gestational age) was obtained by caesarean operation and the articular cartilage was prepared as described above. The dog-fish (Scylliorhinus canicula) 40−50 cm length were obtained from Concarneau, France, and mandibular cartilage was prepared as described above.

b) Extraction of proteoglycans — Cartilage pieces were extracted in 10-times their weight of 4 M guanidine hydrochloride in 0.05 M sodium acetate (pH 5.8) containing 0.01 M EDTA, 0.1 M 6-aminohexanoic acid, 5 mM benzamidine HCl and 10 mM N-ethylmaleimide for 48 h at 4°C. The extracts were filtered through glass wool.

c) Purification of proteoglycans by equilibrium density gradient procedures was performed as described elsewhere (5). One step purification of proteoglycans, under dissociative conditions in 4 M guanidinium chloride, with added CsCl to give a final density of 1.50 g/ml, was performed by centrifugation in a Beckman 50.2 Ti rotor (130 000 xg, 48 h, 18°C, 0.8−1 mg/ml uronic acid starting concentration). At the end of the run centrifuge tubes were frozen in liquid nitrogen and cut into three fractions. The bottom fraction ($D_1 \geq 1.60$) containing 90% of the total hexuronic acid was dialyzed and freeze-dried. Two step purification of proteoglycans was performed as follows: centrifugation under associative conditions was performed after reagreggation of the extracts in 0.4 M guanidinium chloride and addition of CsCl to a density of 1.65 g/ml (130 000 xg, 48 h, 18°C, Beckman type 50.2 Ti rotor, 0.8−1 mg-ml uronic acid starting concentration). The A_1 bottom fraction ($\rho \geq 1.82$ mg/ml) was dialyzed and freeze dried. The A_1 fraction was submitted to centrifugation in a second gradient under dissociative conditions performed as described above the bottom fraction (A_1 $D_1\rho \geq 1.60$) was dialyzed and freeze-dried.

d) Fractionation of D_1 proteoglycans — A sample of D_1 proteoglycans was fractionated according to size by gel chromatography on Sepharose CL−2B in 4 M guanidine hydrochloride, 0.05 acetate buffer pH 5.8. Two subfractions were collected: the first corresponding to the main peak and the second corresponding to a more retarded shoulder (Fig. 1). The subfractions were dialyzed against acetate and then water, freeze-dried and analyzed by gel electrophoresis.

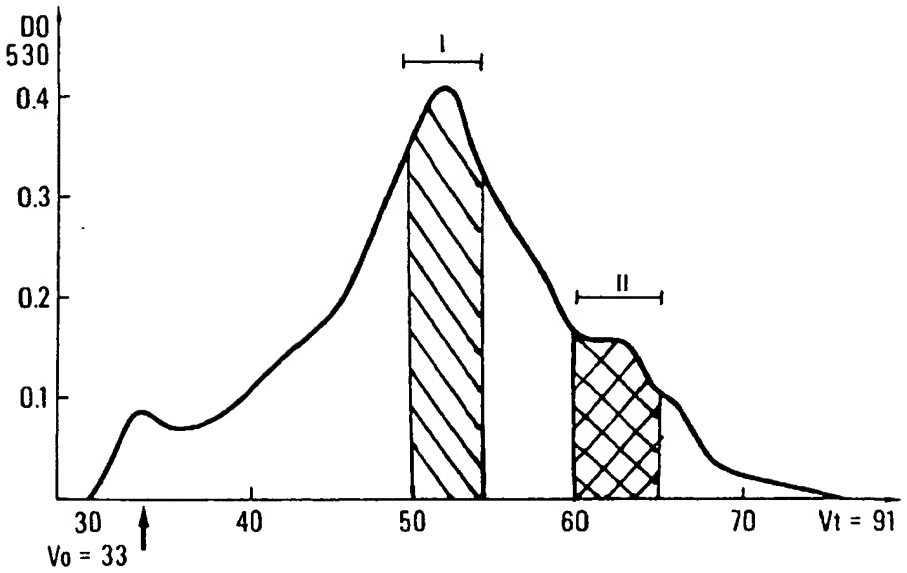

FIGURE 1 Gel chromatography of the D_1 proteoglycan fraction from baboon articular cartilage. Sepharose CL-2B colkumn in 4M guanidine hydrochloride. The pooled fractions I and II were submitted to gel electrophoresis (see Fig. 3).

e) Preparation of the small proteoglycan species of cartilage — A small proteoglycan with a high electrophoretic mobility was identified in various cartilages (4,5,8). The small proteoglycan was isolated from baboon articular cartilage with the method already described by Stanescu and Sweet (10) with small modifications. Material from the top of an associative gradient (A_3, $\rho \leq 1.49$ g/ml) was applied to ion-exchange chromatography columns of DEAE-cellulose Cl^- form) that had been equilibrated with 8M urea in 0.05M Tris/HCl buffer pH 6.8 as described before (5,10). Columns were eluted with 3 bed-volumes each of 0.2M NaCl in 8M urea, 0.3M NaCl in 8M urea and 2M NaCl in 8M urea all in 0.05M Tris/HCl buffer, pH 6.8. The 2M NaCl fraction was dialyzed and freeze-dried. The material was then submitted to gel filtration on Sepharose 4B in the presence of 0.1% SDS. The peak with a K_d 0.43 was reduced and purified again on DEAE-cellulose as described above. The fraction obtained was examined by gel electrophoresis on polyacrylamide-agarose rod gels. SDS-PAGE in various acrylamide concentrations was performed and no protein band was found contaminating the small proteoglycan (10).

Electrophoresis on horizontal submerged polyacrylamide-agarose gels

The various preparations described above were submitted to gel electrophoresis in a Mini-gel electrophoresis system (CBL Uniscience, Cambridge Biotechnology Laboratories).

a) Gel preparation was performed as described by Mc Devitt and Muir (3) with small modifications. The buffer was 0.04M Tris acetate 1mM sodium sulfate

(final concentrations) adjusted at 4°C to pH 6.8 with acetic acid. The gels consisted of 1.2% acrylamide and 0.7% agarose and were prepared as follows Agarose (Indubiose A37) 280 mg was added to 22.5 ml of the buffer in a flask fitted with a condenser, stirred and heated in a boiling water bath for about 20 min, then transferred to a water bath at 40°C with stirring. At the same time, a solution containing 456 mg acrylamide, 24 mg N,N' methylenebisacrylamide, 4.8 ml of 6.4% (v/v in H_2O) β-dimethylaminoproprionitrile and 9.7 ml of the Tris buffer was mixed thoroughly and warmed to 40°C. The acrylamide solution was added to the agarose solution with stirring and to the mixture 3 ml of freshly prepared 3% (W/v) ammonium persulfate in water was added. The final mixture was stirred vigorously for several seconds and 14 ml were poured in the electrophoresis chamber. Two chambers can be filled with the quantity prepared as described above.

b) Electrophoretic conditions and band localization — A Film Gel Bond (FMC corporation, Marine Colloids Division) was put in the electrophoresis chamber and the buffer plates and the applicable comb were positioned.

14 ml of gel were poured and allowed to set at 4°C for 3–4 minutes (gel thickness about 1.8 mm, gel surface 10 × 7.8 cm). 4.5 ml of 0.04 M Tris acetate buffer pH 6.8 were carefully added covering the gel. The gel was kept 3 h at room temperature and over night at 4°C. The buffer was removed and the slots (9 mm long/1 mm wide) were filled with 5–7 µl 8 M urea pH 7 containing 2.5–5.5 µg proteoglycans (dry weight). Then 2 µl 0.01% bromphenol, 50% sucrose was added to each slot and 45 ml of 0.04 M Tris-acetate buffer pH 6.8 were carefully poured. A 15 min premigration (50 mA, 36 v) and a 55 min migration (65 mA, 48 V) were carried out at 4°C. The gels were stained with toluidine blue 0.2% in 0.1 N acetic acid for 7 min and destained in 0.1 N acetic acid for 10 h. They were kept in water at 4°C. The gels were dried at 37°C on the Gelbond Film.

RESULTS AND DISCUSSION

The proteoglycan preparations we analyzed by electrophoresis on horizontal submerged polyacrylamide-agarose gels contained several proteoglycan monomers which differ as regards electrophoretic mobility on cylindrical rod gels and as regards hydrodynamic size on gel chromatography.

The D_1 and A_1D_1 preparations of young baboon articular cartilage proteoglycan yields two metachromatic bands, close one to another, when examined by electrophoresis on cylindrical rod gels (5). The same pattern was obtained on horizontal submerged gels (Fig. 2 lane 2 and lane 6). The bands correspond to two proteoglycan monomers which differ as regards buoyant density in CsCl gradients, the high density correlating with low electrophoretic mobility (5). The two monomers have different K_d when examined by gel filtration on Sepharose CL-2B in 4 M guanidinium chloride (Stanescu and Chaminade, unpublished results). They are similar to the two major monomers of the bovine nasal septum with apparent M_r of 3.5×10^6 and 1.3×10^6. Each of these monomers shows only one band on polyacrylamide-agarose rod gels, the larger having a lower mobility than the slower. The first monomer is chondroitin sulfate rich, whereas the second is keratan sulfate rich (12).

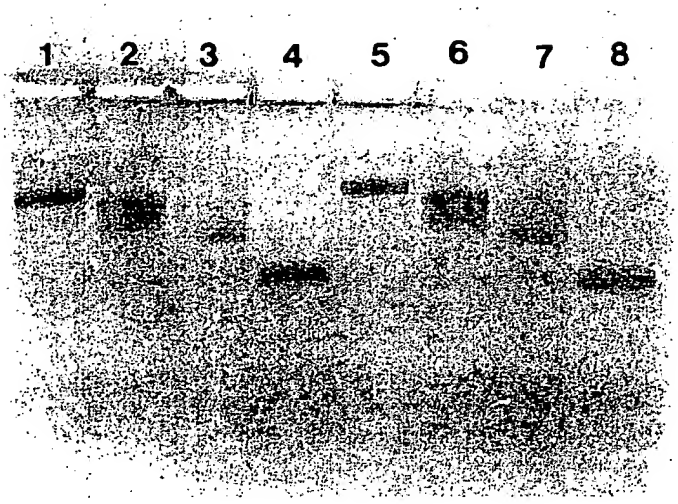

FIGURE 2 Electrophoresis on horizontal submerged polyacrylamide-agarose gels of proteoglycan preparations. Lane 1, D_1 fraction of proteoglycan from baboon fetal cartilage; lane 2, D_1 fraction of proteoglycans from baboon articular cartilage; lane 3, small proteoglycan species of baboon articular cartilage prepared as described in Methods; lane 4, chondroitin sulfate; lane 5, D_1 fraction of proteoglycans from baboon fetal cartilage; lane 6, A_1D_1 fraction from baboon articular cartilage; lane 7, small proteoglycan species of baboon articular cartilage prepared as described in Methods; lane 8, chondroitin sulfate.

The two subfractions of a D_1 sample obtained by gel chromatography as described in the Methods section (Fig. 1) yielded single bands when analyzed by electrophoresis on slab gels. The less retarded proteoglycans gave a slower migrating band than that yielded by the more retarded fraction (Fig. 3, lanes 3 and 4). The same results were obtained by electrophoresis on rod cylindrical gels.

The population of small proteoglycans (prepared from A3 by DEAE chromatography) gave on rod cylindrical gels a thin band which migrated more rapidly than the main monomers (4,5,9,10). Similar results were obtained by electrophoresis on horizontal submerged slab gels (Fig. 2 lanes 3 and 7). The population of small proteoglycan monomer was isolated and characterized from baboon articular cartilage (10). Its hydrodynamic size was smaller than that of the main monomers and its protein content was higher. Similar small proteoglycan monomers were isolated and characterized from bovine nasal septum (11) and from bovine articular cartilage (17) and in the latter study two small proteoglycans were shown to contain dermatan sulfate (17).

The main monomers of the human and baboon fetal cartilage of the last trimester of gestation gave on electrophoresis on rod cylindrical gels a single main band whose migration was slower than that of the main bands of young postnatal cartilages (4) (13) (18). Similar results were obtained by electrophoresis on horizontal submerged slab gels (Fig. 2 lanes 1 and 5). Examined by gel chromatography the human and baboon fetal monomer has a higher hydrodynamic size than the main monomers of young humans and baboons (Stanescu, unpublished

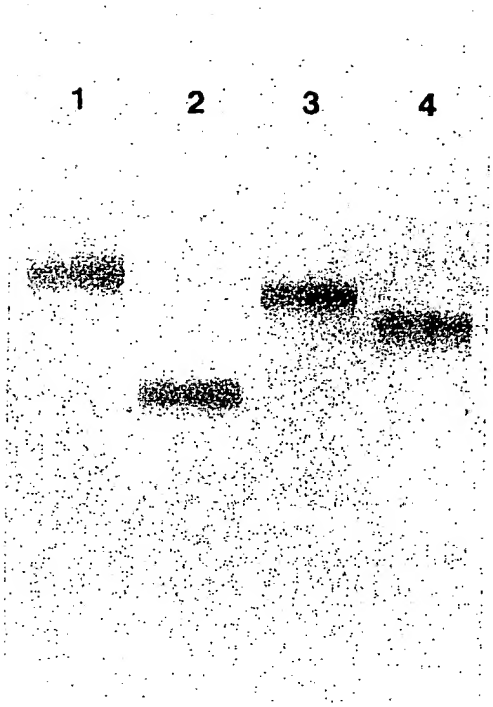

FIGURE 3 Electrophoresis on horizontal submerged polyacrylamide-agarose gels of proteoglycan preparations. Lane 1, D_1 fraction of proteoglycans from dogfish cartilage; lane 2, chondroitin-sulfate; lane 3, subfraction I of D_1 proteoglycans of baboon articular cartilage (see Fig. 1); lane 4, subfraction II of D_1 proteoglycans of baboon articular cartilage (see Fig. 1).

results). The proteoglycan monomers of bovine fetuses are also larger than those from bovine nasal cartilage (19).

The proteoglycan monomers of dog-fish cartilage are remarkable for their large size as demonstrated by gel chromatography, velocity gradient centrifugation and electron microscopy (20). On acrylamide-agarose rod cylindrical gels they yielded a single slow moving band (9) (20) and similar results were obtained on slab submerged gels (Fig. 3 lane 1).

These results show that the separation of proteoglycan monomers by electrophoresis on submerged polyacrylamide-agarose gels is similar to that previously obtained on rod gels (4–9). The methodical procedure of the cylindrical gel rods is more tedious and troublesome compared to the very simple horizontal submerged separation system. Thus particularly in laboratories where many samples are to be analyzed (e.g. column fractions) the latter method is more suitable. The method may also be useful if electrophoretic transfer from gels to membranes is needed.

REFERENCES

1. A. C. Peacock and C. W. Dingam, *Biochemistry*, 7, 668 (1968).
2. A. E. Dahlberg, C. W. Dingman and A. C. Peacock, *J. Mol. Biol.*, 41, 139 (1969)
3. C. A. Mc Devitt and H. Muir, *Anal. Biochem.* 44, 612 (1971).
4. V. Stanescu, P. Maroteaux and E. Sobczak, *Biomedicine*, 19, 460 (1973).
5. V. Stanescu, P. Maroteaux and E. Sobczak, *Biochem. J.*, 163, 103 (1977).
6. J. P. Pearson and R. M. Mason, *Biochim. Biophys. Acta*, 498, 176 (1977).
7. M. T. Bayliss and S. Y. Ali, *Biochem. J.*, 176, 683 (1978).
8. V. Stanescu, P. Maroteaux and E. Sobczak, *Biochim. Biophys. Acta*, 629, 371 (1980).
9. V. Stanescu and R. Stanescu, *Biochim. Biophys. Acta*, 757, 377 (1983).
10. V. Stanescu and M. B. E. Sweet, *Biochim. Biophys. Acta*, 673, 101 (1981).
11. D. Heinegard, M. Paulsson, S. Inerot and C. Carlström, *Biochem. J.*, 197, 355 (1981).
12. D. Heinegard, J. Wieslander, J. Sheehan, M. Paulsson and Y. Sommarin, *Biochem. J.*, 225, 95 (1985).
13. V. Stanescu and P. Maroteaux, *Pediatr. Res.*, 9, 779 (1975).
14. V. Stanescu, R. Stanescu and P. Maroteaux, *J. Bone Joint Surg.*, 66–A, 817 (1984).
15. M. B. E. Sweet, E. J. -M. A. Thonar and A. R. Immelman, *Arch. Biochem. Biophys.*, 189, 28 (1978).
16. S. Inerot, D. Heinegard, *Collagen Rel. Res.*, 3, 245 (1983).
17. L. C. Rosenberg, H. U. Choi, L. H. Tang, T. L. Johnson, S. Pal, C. Weber, A. Reiner and A. R. Poole, *J. Biol. Chem.*, 260, 6304 (1985).
18. V. Stanescu, P. Maroteaux, R. Stanescu and E. Sobczak, *Biol. Neonate*, 27, 361 (1975).
19. S. Pal, L. H. Tang, H. Choi, c. Habermann, L. Rosenberg, P. Roughley and A. R. Poole, *Collagen Rel. Res.*, 2, 151 (1981).
20. E. J. -M. A. Thonar, V. Stanescu, M. B. E. Sweet, J. Buckwalter, J. Pita and K. E. Kuettner, *Trans. Orthop. Res. Soc.*, 10, 57 (1985).

Note Added in Proof. Since our manuscript was submitted a vertical slab gel electrophoresis method for proteoglycans was published (Heinegard et al., *Anal Biochem.* 151, 41, 1985).